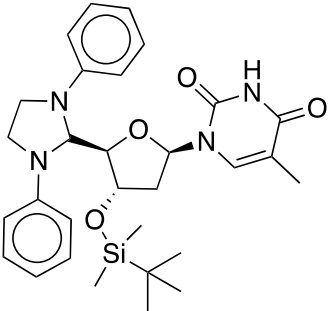
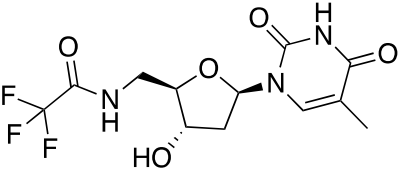
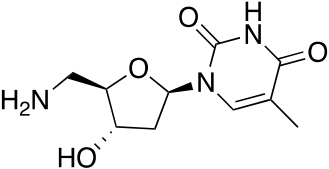
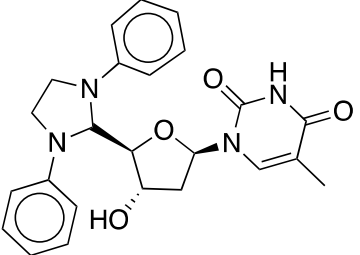
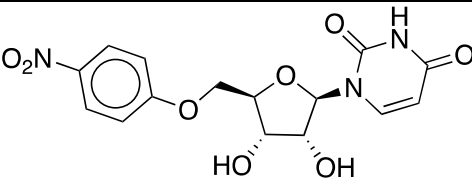
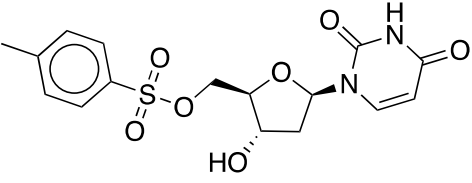
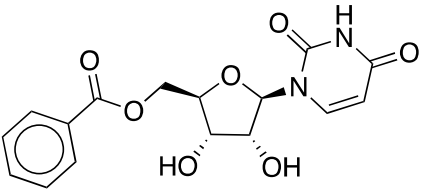
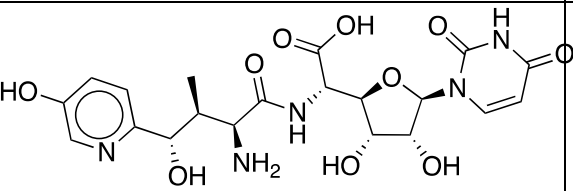


Supplementary Material

Table S1: Structure and nomenclature of hyaluronan synthase inhibitors

Compound	Structure	Name	Classification
1		3'-O-(TBDMS)-5'-Deoxy-5'-(1,3-Diphenyl-2-Imidazolidinyl)-Thymidine	Thymidine Analog
2		5'-Trifluoroacetamido-5'-Deoxythymidine	Thymidine Analog
3		5'-Amino-5'-Deoxythymidine	Thymidine Analog

4		5'-Deoxy-5'-(1,3-Diphenyl-2-Imidazolidinyl)-Thymidine DDIT	Thymidine Analog
5		5'-O-(4-Nitrophenyl)-Uridine	Uridine Analog
6		2'-Deoxy-5'-O-p-Toluenesulfonyluridine	Uridine Analog
7		5'-O-Benzoyluridine	Uridine Analog
8		Nikkomycin Z	Natural Product (Uridine Analog)

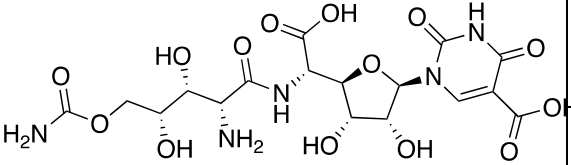
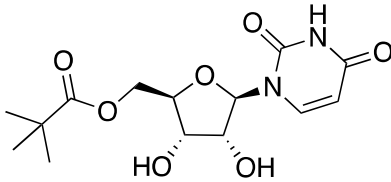
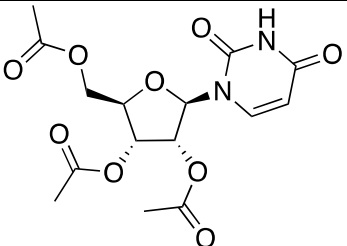
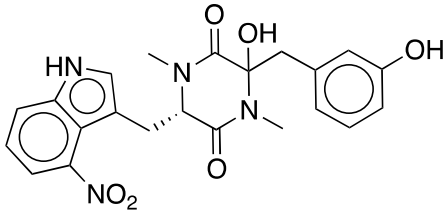
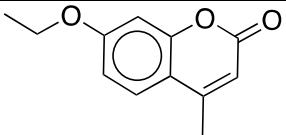
9		Polyoxin D	Natural Product (Uridine Analog)
10		5'-O-Pivaloyluridine	Uridine Analog
11		Uridine Triacetate	Uridine Analog
12		Thaxtomin A	Cellulose Synthesis Inhibitor
13		Morlin	Cellulose Synthesis Inhibitor

Table S2: Vendor information of the compounds used in the present study.

Compound	IUPAC Name	Vendor	CAS#
1	1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(1,3-diphenylimidazolidin-2-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione	BERRY & ASSOCIATES	149741-58-2
2	2,2,2-trifluoro-N-(((2R,3S,5R)-3-hydroxy-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl)acetamide	BERRY & ASSOCIATES	55812-00-5
3	1-((2R,4S,5R)-5-(aminomethyl)-4-hydroxytetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione	BERRY & ASSOCIATES	25152-20-9
4	1-((2R,4S,5R)-5-(1,3-diphenylimidazolidin-2-yl)-4-hydroxytetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione	BERRY & ASSOCIATES	869355-30-6
5	1-((2R,3R,4S,5R)-3,4-dihydroxy-5-((4-nitrophenoxy)methyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione	AKOS	39946-91-3
6	((2R,3S,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl 4-methylbenzenesulfonate	CARBOSYNTH	27999-47-9
7	((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-	LIFE CHEMICALS	54618-06-3

	dihydroxytetrahydrofuran-2-yl)methyl benzoate		
8	(S)-2-((2S,3S,4S)-2-amino-4-hydroxy-4-(5-hydroxypyridin-2-yl)-3-methylbutanamido)-2-((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)acetic acid	SIGMA	59456-70-1
9	1-((2R,3R,4S,5R)-5-((S)-((2R,3R,4R)-2-amino-5-(carbamoxyloxy)-3,4-dihydroxypentanamido)(carboxy)methyl)-3,4-dihydroxytetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid	EMD CHEMICALS	22976-86-9
10	((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl pivalate	AMBINTER	6554-06-9
11	(2R,3R,4R,5R)-2-(acetoxymethyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3,4-diyl diacetate	ARK PHARM	4105-38-8
12	(6S)-3-hydroxy-3-(3-hydroxybenzyl)-1,4-dimethyl-6-((4-nitro-1H-indol-3-yl)methyl)piperazine-2,5-dione	SANTA CRUZ	122380-18-1
13	7-ethoxy-4-methyl-2H-chromen-2-one	SIGMA	87-05-8

Table S3: RT-qPCR primer sequences used in the present study.

Target gene	Primer Sequence (5'-3')	T _{Annealing}
<i>CD44s</i>	Sense: ATAATAAAGGAGCAGCACTTCAGGA Anti-sense: ATAATTTGTGTCTTGGTCTCTGGTAGC	60°C
<i>CD44v3</i>	Sense: ATAATGGCTGGGAGCCAAATGAAGAAA Anti-sense: ATAATCATCATCATCAATGCCTGATCCAGA	60°C
<i>CD44v6</i>	Sense: ATAATCAGAAGGAACAGTGGTTTGGCA Anti-sense: ATAATGTCTTCTTTGGGTGTTTGGCGA	60°C
<i>CD44v9</i>	Sense: ATAATGAGCTTCTCTACATCACATGAAGGC Anti-sense: TAATGTCAGAGTAGAAGTTGTTGGATGGTC	60°C
<i>RHAMM</i>	Sense: GCAAACACTGGATGAGCTTGA Anti-sense: TGGTCTGCAGATCTAGAAGCA	
<i>HAS1</i>	Sense: GGAATAACCTCTTGCAGCAGTTTC Anti-sense: GCCGGTCATCCCCAAAAG	60°C
<i>HAS2</i>	Sense: TCGCAACACGTAACGCAAT Anti-sense: ACTTCTCTTTTTCCACCCCATTT	60°C
<i>HAS3</i>	Sense: AACAAAGTACGACTCATGGATTTCTT Anti-sense: GCCCGCTCCACGTTGA	60°C
<i>HYAL-1</i>	Sense: GATTGCAGTGTCTTCGATGTGGTA Anti-sense: GGGAGCTATAGAAAATTGTCATGTCA	60°C
<i>HYAL-2</i>	Sense: CTAATGAGGGTTTTGTGAACCAGAATAT Anti-sense: GCAGAATCGAAGCGTGGATAC	60°C
<i>TMEM2</i>	Sense: GGAATAGGACTGACCTTTGCCAG Anti-sense: TTCTGACCACCCTGAAAGCCGT	60°C
<i>KIAA1199/CEMIP</i>	Sense: ACCGAGCACATTCCAACCTACCG Anti-sense: GGCAGAGATGATTGAGAGGAACG	60°C

<i>OCT4</i>	Sense: AGTGCCCGAAACCCACACT Anti-sense: CTTCTGGCGCCGGTTACA	60°C
<i>NANOG</i>	Sense: TGCCTCACACGGAGACTGTCT Anti-sense: AGTGGGTTGTTTGCCTTTGG	60°C
<i>SOX2</i>	Sense: ACACCCTGATCTGGCATGGA Anti-sense: GGCTGTTGCCTGGCTTCTC	60°C
<i>TBP</i>	Sense: TGGCGTGTGAAGATAACCCAA Anti-sense: TCTTGGCAAACCAGAAACCCT	60°C

Table S4: Antibodies used in the present study.

Antigen	Specie	Catalog No.	Dilution
CD44	Mouse	Hermes-3, kindly provided by Dr. Jalkanen	1 µg/mL
HAS2	Mouse	Santa Cruz Biotechnology, Inc, A7, #sc-514737	1:200
c-myc	Mouse	Santa Cruz Biotechnology, Inc, 9E10, #sc-40	1:200
Cyclin B1	Rabbit	Cell Signaling, #4138	1:1,000
Cyclin E1	Mouse	Cell Signaling, #4129	1:1,000
Cyclin D1	Mouse	Santa Cruz Biotechnology, Inc, A-12, #sc-8396	1:200
Caspase-3	Rabbit	Cell Signaling, #9665	1:1,000
phospho-S6 (Ser235/236)	Rabbit	Cell Signaling, #2211	1:1,000
S6	Mouse	Cell Signaling, #2317	1:1,000
p27	Rabbit	Cell Signaling, #3686	1:1,000
GAPDH	Rabbit	Cell Signaling, 14C10, #2118	1:2,000
Anti-mouse IgG (H+L) Secondary antibody, HRP	Goat	ThermoFischer Scientific, #31430	1:10,000
Anti-rabbit IgG (H+L) Secondary antibody, HRP	Goat	ThermoFischer Scientific, #31460	1:10,000

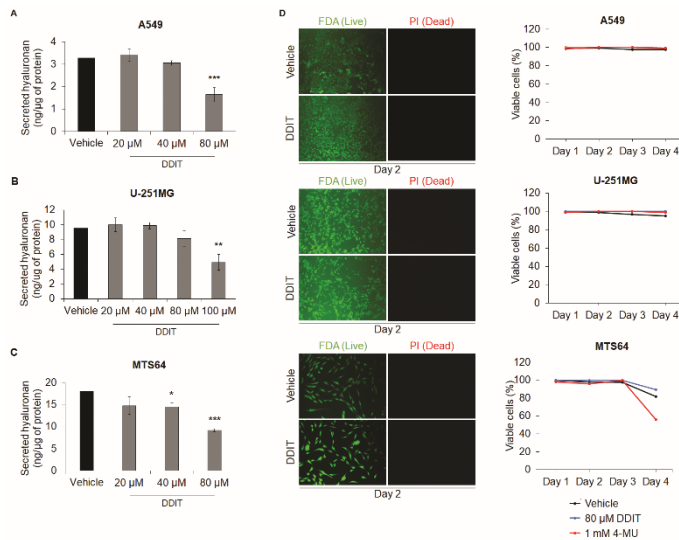


Figure S1: Dose-response analysis of the effect of DDIT on hyaluronan synthesis and toxicity. Hyaluronan secreted by A549 lung adenocarcinoma cells (A), U-251MG glioblastoma cells (B) and MTS64 normal dermal fibroblasts (C) after treatment with vehicle (0.04% DMSO) or DDIT (20, 40, 80 and 100 μM) in serum-free medium, for 24 hours. (E) Viability of A549 lung adenocarcinoma cells, U-251MG glioblastoma cells and MTS64 normal dermal fibroblasts was determined by FDA/PI staining of live (green) or dead (red) cells, after treatment with vehicle (0.04% DMSO), or DDIT (80 μM) in serum-free medium, for 1, 2, 3 and 4 days. Images show representative staining of the respective cell lines from day two. The images were captured with a 5x objective.

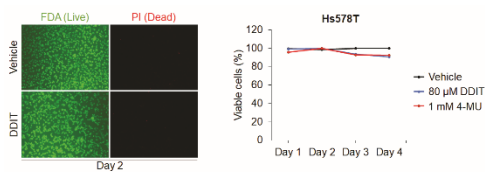


Figure S2: Viability of Hs578T cells. The viability of the cells was determined by FDA/PI staining of live (green) or dead (red) cells, after treatment with 100 μM DDIT .

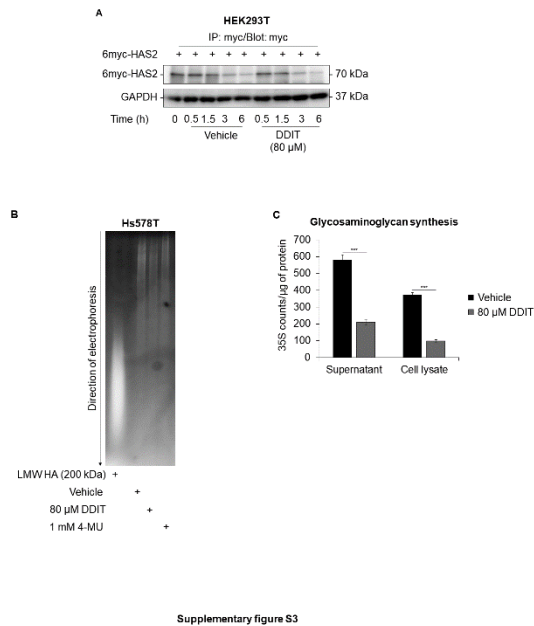


Figure S3: Effect of DDIT on 6myc-HAS2 stability, size of hyaluronan, and glycosaminoglycan synthesis. HEK293T cells were transfected with 6myc-tagged HAS2 for 24 hours. Then, lysates from untreated or treated cells with 20 μ M cycloheximide (for different time points) were subjected to immunoprecipitation followed by protein separation on SDS-PAGE (A). The size of hyaluronan in 24 h conditioned media of untreated or DDIT- and 4-MU-treated cultures was analyzed by agarose gel electrophoresis (B). Ion-exchange chromatography of Hs578T cells treated or not with DDIT as described in Materials and Methods.

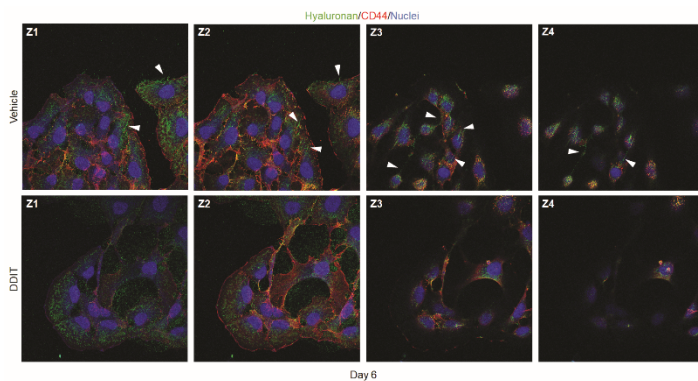


Figure S4: DDIT inhibits formation of hyaluronan cable-like structures. Z-stacks of confocal imaging of Hs578T breast cancer cells stained for hyaluronan (green) and CD44 (red) after treatment with vehicle (0.04% DMSO) or DDIT (80 μ M) in 10% FBS, for 6 days. Arrow heads indicates hyaluronan cable-like structures.

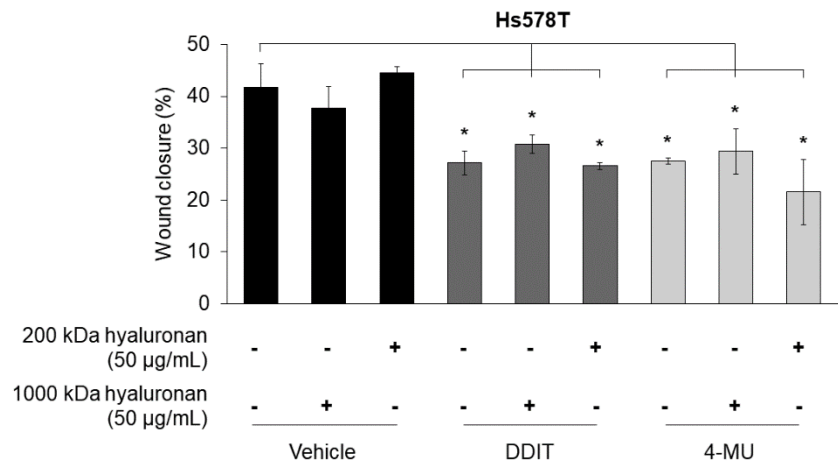


Figure S5: Exogenous hyaluronan does not recover breast cancer cell migration after hyaluronan synthesis inhibition. Wound healing of Hs578T breast cancer cells after treatment with vehicle (0.04% DMSO), DDIT (80 µM), 4-MU (1 mM) and 1000 or 200 kDa exogenous hyaluronan (50 µg/mL) in 10% FBS, for 12 hours.

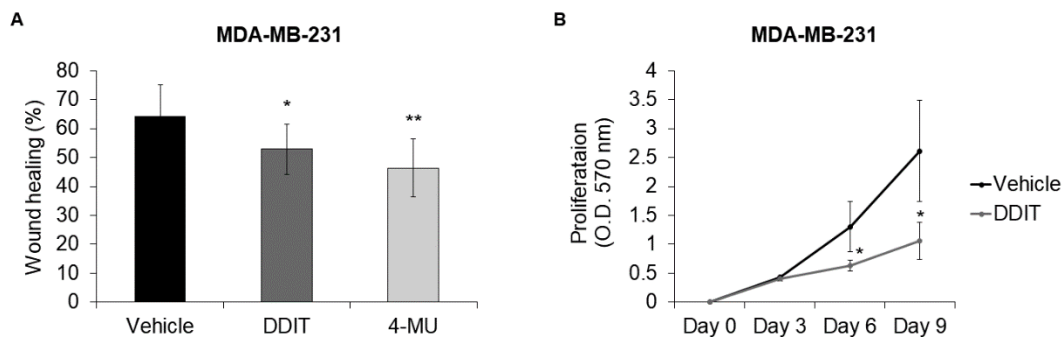


Figure S6: DDIT inhibits migration and proliferation of MDA-MB-231. (A) Wound healing of MDA-MB-231 breast cancer cells after treatment with vehicle (0.04% DMSO), DDIT (80 µM) or 4-MU (1 mM) in medium containing 10% FBS for 24 hours. (B) Proliferation of MDA-MB-231 breast cancer cells after treatment with vehicle (0.04% DMSO) or DDIT (80 µM) for 3, 6 and 9 days, in medium containing 10% FBS.

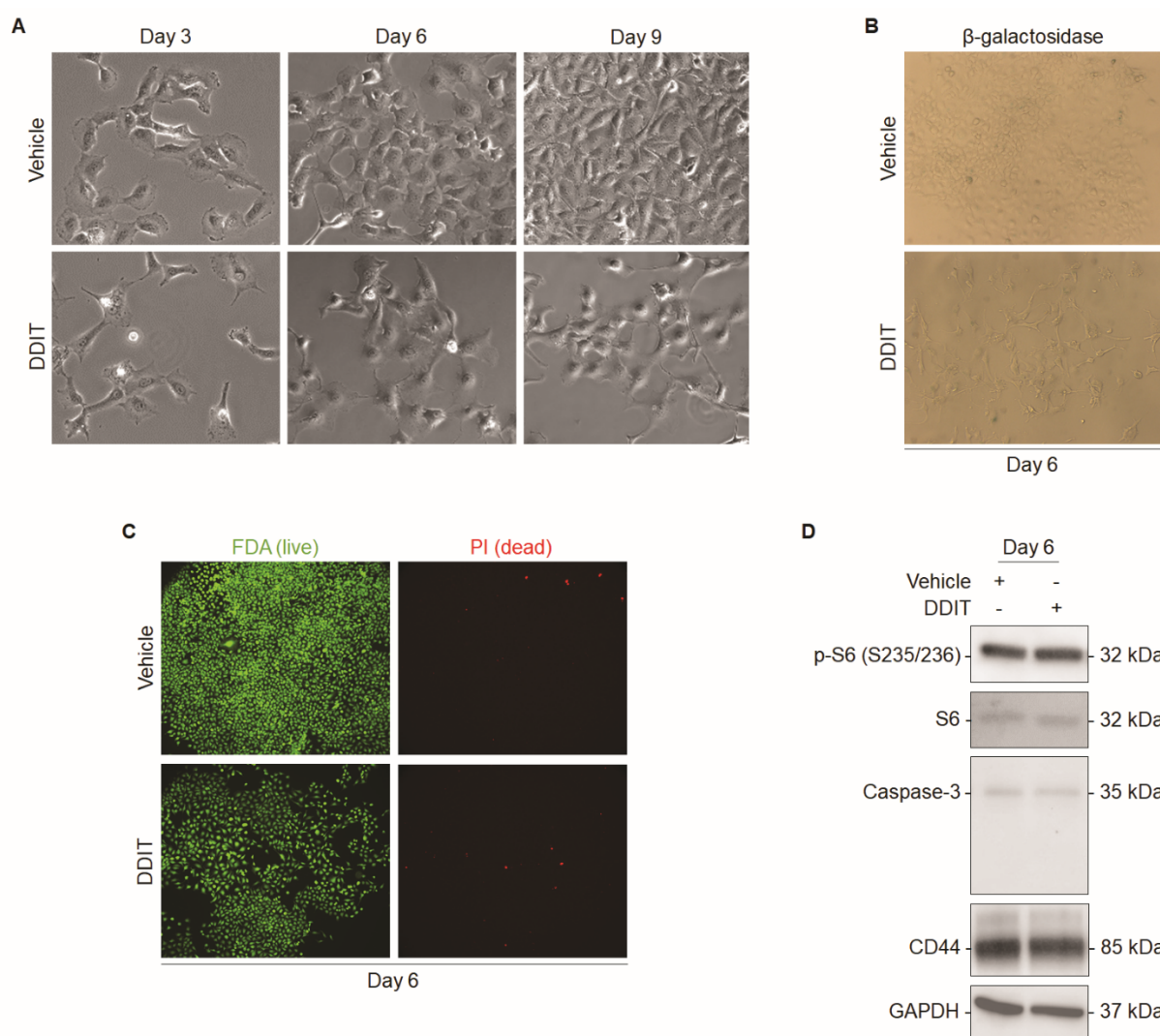


Figure S7: Long-term treatment with DDIT does not induce senescence or apoptosis in breast cancer cells. (A) Phase contrast images of Hs578T cells after 3, 6 and 9 days of treatment with vehicle (0.04% DMSO) or DDIT (80 μ M) in medium containing 10% FBS and high glucose concentration. The images were captured with a 10X objective. (B-C) β -galactosidase (B) and FDA/PI (C) staining of live (green) or dead (red) Hs578T cells after 6 days of treatment with vehicle or DDIT in 10% FBS high glucose medium. The images were captured with a 5x objective. (D) Immunoblotting for p-S6 (S235/236)/S6, caspase-3, CD44 and GAPDH in total cell lysates of Hs578T after treatment with vehicle or DDIT for 6 days, in medium supplemented with 10% FBS.

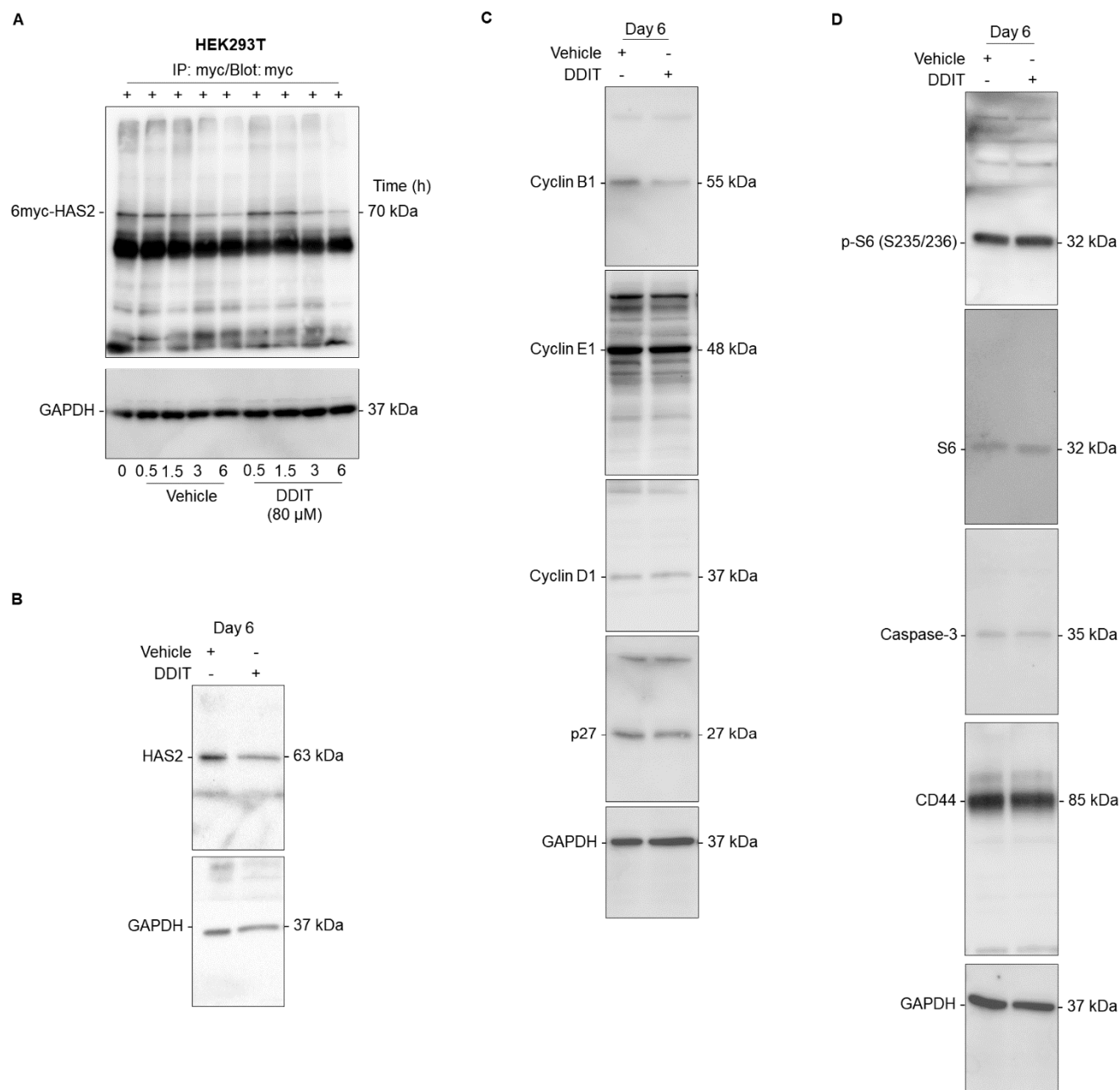


Figure S8: Uncropped blots corresponding to (A) Fig. S3A, (B) Fig. 8C, (C) Fig. 8E and (D) Fig. S7D.